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Earlier results from this project suggested that the prognosis for patients with node-negative ductal breast carcinomas was poor, when tumor cells expressed the cell-surface Le^a-Le^x cell-surface oligosaccharide. However, the statistical significance of this finding was lost, when results obtained during the past year analyzing tumor specimen from the last 95 patients of this study were included in the statistical analysis. Certain technical problems were encountered during the immunohistochemical analysis of this last set of patients and we are currently attempting to correct these and to re-analyze duplicate specimens. Analysis of the cell surface oligosaccharides Le^a, Tn, T antigen, sialyl- Le^a, and Le^x showed no significant correlations with prognosis. Other related studies led to the identification of two new cDNA clones whose cognate genes are overexpressed in Le^a-Le^x positive cancer cells. Partial DNA sequencing has indicated that these clones are partially homologous to known human fucosyltransferases. The cognate genes for these clones are candidates for those that are aberrantly expressed in cancer cells that are Le^a-Le^x positive.

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David E. Bellezza 9/28/98

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TABLE OF CONTENTS

ANNUAL REPORT FOR GRANT NUMBER DAMD17-94-J-4363

INTRODUCTION

Patients who have small node-negative ductal breast carcinomas generally have a favorable prognosis (see ref. 1 for review). After surgery, relapse occurs in less than 20% of these so called low-risk patients during the following 10 year period. In spite of this favorable prognosis, the management of these low-risk patients can be complicated, as there is no established way to identify the 20% who will relapse. The patients who could benefit most from adjuvant chemotherapy cannot be reliably predicted and equally important, the patients who don't require post-surgical adjuvant therapy cannot be identified. The purpose of the present research is to devise an approach exploiting unusual oligosaccharide cell-surface markers that appear on breast cancer cells to identify the low-risk patients who are at risk. A further goal is to determine which genes are involved in the aberrant expression of the unusual oligosaccharides found on some but not all cells of ductal breast carcinomas.

The overall approach of these studies is to determine if there are specific combinations of oligosaccharide markers and other makers on breast cancer cells that are useful in predicting the post surgical prognosis of low-risk node-negative breast cancer patients. The markers identified from these studies would then be combined with other known prognostic markers in an attempt to assemble a set of markers which could indicate with highest specificity and sensitivity the patients who are at greatest risk for relapse. The studies are also intended to identify glycosyltransferase activities that may be expressed in certain carcinomas that are correlated with poor prognosis. This identification would open the way for new approaches to studying the biological effects of the most significant oligosaccharides.

A large group of breast tumor specimen was obtained from a collection of the Danish Breast Cancer Cooperative Group, which is a nationwide surveillance and research program (2). All specimen are from women who had low-risk node negative ductal breast carcinomas and who had surgery 5-20 years previously and who have been closely followed since surgery. None of the women had chemotherapy, so that the prognosis is unaffected by other post-surgical interventions. A panel of well-characterized monoclonal antibodies with known specificity for specific oligosaccharides is employed to define the cell surface oligosaccharides, proteolytic activities (such as Cathepsins) and protease inhibitors associated with the tumor cells. After completing the survey, the relapse history of the patients will be compared with the different molecular markers using Cox's proportional hazards model (3) to identify statistically significant independent markers of prognosis. It will then be possible to select different combinations of markers to attempt to improve specificity and sensitivity by using a panel of prognostic markers.

Further research is identifying the glycosyltransferase activities that are abnormally expressed in breast cancer cells that lead to aberrant expression of specific marker oligosaccharides. Here we are cloning cDNAs recognizing genes that are expressed in cells overexpressing the Le^a-Le^x oligosaccharide, which is at this time the best prognostic indicator, which we have identified. We are also beginning studies of the effects of Le^a-

Le^x cell-cell interactions in carcinomas.

The research is still in progress.. Therefore conclusions and detailed summaries of the data to date are premature. However the preliminary review of the data provided below indicates that there could be a statistically significant association of the Le^a-Le^x oligosaccharide and poor prognosis of low-risk ductal breast carcinomas.

BODY

We continue to use the panel of monoclonal antibodies (Mabs) specific for the designated oligosaccharides and in the last year have completed the application of the entire panel to multiple paraffin sections of the total tumor specimens from 181 low-risk ductal breast carcinoma patients. As noted in the last progress report, this number is somewhat less than the original number stated in our initial proposal, since our Danish colleagues in Odense informed us that they have had to remove some of the specimens from the study because: a) Certain specimens were exhausted and now contained only surrounding normal tissue; b) Re-examination of clinical data revealed that some patients had received chemotherapy and therefore could not be included in this study; c) Specimens were eliminated because later data showed the patients were node-positive and therefore could not be included..

As in the previous year, we used double-label immunofluorescence microscopy techniques that apply fluorescene and rhodamine conjugated antibodies simultaneously so that the distribution of two different oligosaccharides can be simultaneously determined in the same tumor section (4,5). The Quantimet 500+ Image Processing System was used to analyze fluorescence images and to define both the fraction of tumor cells that are positive, (above a defined baseline), and the intensity of the reaction relative to positive and negative control cells that are processed at the same time. The fraction of positive tumor cells and the relative amounts of each cell surface component on the tumor cells is therefore determined.

There is ongoing statistical analysis at the Biostatistics Core Laboratory of the University of Colorado Cancer Center of the 181 tumor specimen that have been competed. We are analyzing both single markers, multiple markers in combinations, and attempting a protocol for the analysis of the ratios of makers in attempts to sharpen prognostic indications of the multiple markers. The statistical analysis is using the proportional hazards model of Cox (6). We have found no significant association of Cathepsin D expression, nor of Le^a, T antigen, Tn antigen, sialy-Le^a, or Le^x with the prognosis of low-risk ductal breast carcinomas (task 2). As described in previous last progress reports, the analysis of the Le^a-Le^x marker alone on the first 86 tumor specimen showed statistically significant ($P<0.005$) correlation with poor prognosis. However when more recent data from the last 95 patients was included in the analysis the statistical significance of the prognostic correlation was lost. We are now seeking further understanding of this confusing change. In analyzing data we discovered that in the last 86 specimen the average intensity of reaction of MAB 43-9F (specific for Le^a-Le^x) with positive tumor cells has markedly declined. During the past 3 months we have therefore

recalibrated all of our immunostaining methods to optimize reactions. With this in hand we are now reexamining additional tumor sections from the last 86 specimen. We will complete this analysis in the next month and therefore complete tasks 1-3 of the project..

As noted in the last progress report in pursuing tasks, 4-5 we obtained two cDNA clones that effect expression of Le^a-Le^x cell-surface oligosaccharides in our test cancer cell line. One codes for previously discovered Decay Accelerating Factor (DAF) and the other named SIL is a previously undiscovered gene. We continue to investigate these clones pursuant to Task 5. In addition a new cDNA library from the mRNA of cell line NU-6-1 has been constructed in the vector pBK CMV (Stratagene). This is an expression vector for mammalian cells. Our screening procedures applied to this library yielded two new positive clones. Partial DNA sequencing showed that both have sequence homology to human fucosyltransferases. One is similar to human alpha (1,3) fucosyltransferase and the other to human alpha (1,3/1,4) fucosyltransferase. Both of these clones were transfected into human cell lines which were previously negative for the Le^a-Le^x cell surface oligosaccharide and the transfectants acquired cell-surface fucosyl groups required for several of our specific MABs. These cDNAs which represent genes heavily expressed in Le^a-Le^x positive cancer cells are candidates for markers of the aberrant gene expression required to develop Le^a-Le^x positive cells. We will complete the sequencing of these clones and deposit the data into GENBANK. Attempts to isolate further glycosyltransferases by expression cloning of this library are ongoing.

The Le^a-Le^x oligosaccharide is heavily associated with mucins in the NU6-1 cell line. We have attempted to produce monoclonal antibodies specific for the protein core of this mucin. NU6-1 cells were grown in regular medium supplemented with [3H] glucosamine and during the last 24-46 hrs grown in serum-free media. The mucins and other products secreted into the medium were precipitated by ammonium sulfate and mucins purified on Sephacryl 400 columns. Purified mucins were deglycosylated using hydrogen fluoride and the purified protein core injected into BALB-C mice for production of MABs. A panel of 10 MABs were selected that may be useful in testing the state of glycosylation of the mucins that normally carry Le^a-Le^x. Preliminary studies of two of these antibodies has shown strong specificties for ovarian cancers and ovarian cysts, while normal ovaries show no reaction using these same MABs.

CONCLUSIONS

Part of our results continue to support the conclusion that the prognosis for low-risk ductal breast carcinomas is poor when the cells are positive for the extended Le^a-Le^x oligosaccharide. The most recent results, however, which are associated with technical difficulties, have clouded this interpretation and we are continuing to confirm the significance of Le^a-Le^x. Analysis of other cell surface oligosaccharides, Le^a, T antigen, Tn antigen sialy-Le^a or Le^x showed no significant correlation with prognosis. Two new cDNAs were cloned coding for what are apparently new human fucosyltransferases that are overexpressed in cancer cells making Le^a-Le^x. These are candidates for genes that are aberrantly expressed in breast cancer cells of low-risk ductal carcinomas having poor prognosis.

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